PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/22, 31/195, 31/155 — A1

(11) International Publication Number: **WO 95/13805**

(43) International Publication Date: 26 May 1995 (26.05.95)

(21) International Application Number: PCT/US94/13239

(22) International Filing Date: 17 November 1994 (17.11.94)

(30) Priority Data:
08/153,469 17 November 1993 (17.11.93) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/153,469 (CIP)
Filed on 17 November 1993 (17.11.93)

(71) Applicant *(for all designated States except US)*: DUKE UNIVERSITY MEDICAL CENTER [US/US]; Erwin Road, Durham, NC 27710 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: WEINBERG, J., Brice [US/US]; 2617 Snow Hill Road, Durham, NC 27712 (US).

(74) Agents: BROOK, David, E. et al.; Hamilton, Brook, Smith & Reynolds, Two Militia Drive, Lexington, MA 02173 (US).

(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF NITRIC OXIDE SYNTHASE INHIBITORS IN THE TREATMENT OF AUTOIMMUNE DISEASES

(57) Abstract

The invention relates to a method of treating or preventing autoimmune diseases, such as rheumatoid arthritis, insulin dependent diabetes mellitus, systemic lupus erythematosus and glomerulonephritis, comprising administering to a patient in need thereof an effective amount of a nitric oxide synthase inhibitor or a nitric oxide scavenger.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**GB**	United Kingdom	**MR**	Mauritania
AU	Australia	**GE**	Georgia	**MW**	Malawi
BB	Barbados	**GN**	Guinea	**NE**	Niger
BE	Belgium	**GR**	Greece	**NL**	Netherlands
BF	Burkina Faso	**HU**	Hungary	**NO**	Norway
BG	Bulgaria	**IE**	Ireland	**NZ**	New Zealand
BJ	Benin	**IT**	Italy	**PL**	Poland
BR	Brazil	**JP**	Japan	**PT**	Portugal
BY	Belarus	**KE**	Kenya	**RO**	Romania
CA	Canada	**KG**	Kyrgystan	**RU**	Russian Federation
CF	Central African Republic	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CG	Congo			**SE**	Sweden
CH	Switzerland	**KR**	Republic of Korea	**SI**	Slovenia
CI	Côte d'Ivoire	**KZ**	Kazakhstan	**SK**	Slovakia
CM	Cameroon	**LI**	Liechtenstein	**SN**	Senegal
CN	China	**LK**	Sri Lanka	**TD**	Chad
CS	Czechoslovakia	**LU**	Luxembourg	**TG**	Togo
CZ	Czech Republic	**LV**	Latvia	**TJ**	Tajikistan
DE	Germany	**MC**	Monaco	**TT**	Trinidad and Tobago
DK	Denmark	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**US**	United States of America
FI	Finland	**ML**	Mali	**UZ**	Uzbekistan
FR	France	**MN**	Mongolia	**VN**	Viet Nam
GA	Gabon				

USE OF NITRIC OXIDE SYNTHASE INHIBITORS IN THE TREATMENT OF AUTOIMMUNE DISEASES

BACKGROUND OF THE INVENTION

MRL-*lpr/lpr* mice have been studied as a model for human autoimmune diseases. This strain of mice develops a spontaneous autoimmune disease characterized by lymphadenopathy, autoantibody production and inflammatory manifestations including nephritis, vasculitis, and arthritis (Hang, L., *et al.*, *J. Exp. Med.* *155*:1690-1701 (1982); Eisenberg, R.A., *et al.*, *Clin. Exp. Rheumatol.* 7:S35-S40 (1989)). Immune function abnormalities also include enhanced constitutive macrophage class II antigen expression, elevated levels of IFN-γ, TNF, IL-1, and IL-6 in isolated kidney, lymph node, and spleen cells, and an enhanced state of macrophage "activation". These disease manifestations are a result of both a single gene mutation *(lpr)* of the *Fas* apoptosis gene on mouse Chromosome 19 and background genes from the MRL strain. Although the MRL genes contributing to disease manifestations have not been identified, two loci contributing to renal disease have been mapped to regions of mouse Chromosomes 7 and 12.

NO (nitric oxide), a multifunctional molecule produced by diverse cell types, results from the conversion of L-arginine to L-citrulline and NO by the action of the enzyme nitric oxide synthase (NOS). NO has been noted to promote relaxation of smooth muscle, serve as a neurotransmitter, cause stasis and/or lysis of microbes and tumor cells, and modulate function and differentiation of hematopoietic cells.

NO also has potent proinflammatory actions. NO may increase vascular permeability in inflamed tissues. Pain is also an important aspect of inflammation. Several groups have now demonstrated that NO plays a role in the

mediation of pain in inflammation. Intradermal injection of solutions containing NO into humans caused a dose-related occurrence of pain in the site (Holthusen, H., and Arndt, J.O., *Neuroscience Letters 165*:71-74 (1994). Thus, pain, a hallmark symptom of inflammation, can be induced by NO.

NO has also been shown to cause increased production of TNF and IL-1 by cells, and to increase the potential of cells to produce hydrogen peroxide. Also, rabbit and human chondrocytes have been shown to produce NO and to express inducible nitric oxide synthase (iNOS) in response to various cytokines and bacterial products. NO is an important mediator in immune complex vasculitis in rats (Mulligan, M.S., *et al., Brit. J. Pharmacol. 107*:1159-1162 (1992)). Some researchers have noted a role for NO in inflammatory bowel disease in guinea pigs (Miller, M.J., *et al., J. Pharmacol. Exp. Ther. 264*:11-16 (1993)), and in induced nephritis in mice or rats (Farrario, R., *et al., J. Am Soc. Nephrol. 4*:1847-1854 (1994)). Research in non-human models of inflammatory diseases has also established that NO participates in experimentally-induced uveitis (Parks, D.J., *et al., Arch. Ophthalmol. 112*:544-546 (1994)).

Studies of humans with arthritis demonstrate further that NO plays an important role in human arthritis. Farrell *et al.* showed that patients with inflammatory arthritis (rheumatoid arthritis and osteoarthritis) had elevated levels of the NO catabolite nitrite in their synovial fluid and serum (Farrell, A.J., *et al., Ann. Rheum. Dis. 51*:1219-1222 (1992)). Patients with rheumatoid arthritis had increased synovial fluid and serum levels of nitrotyrosine (Kaur, H., and Halliwell, B., *Febs Letters 350*:9-12 (1994)).

Cell-derived NO reacts with superoxide ($O_2 \cdot -$) to form peroxynitrite (ONOO-), which in turn may spontaneously produce hydroxyl radical (HO·), a molecule with high potential for cell and tissue injury and destruction. Other investigators have shown in a rat model of inflammation that peroxynitrite is pro-inflammatory (Rachmilewitz, D., *et al.*, *Gastroenterology 105*:1681-1688 (1993)).

Nitrotyrosine is formed by the action of peroxynitrite on tyrosine, and it is felt to be a stable "footprint" or "track" of the presence of NO (Beckman, J.S. *et al.*, *Methods Enzymol. 233*:229-240 (1994)). Nitrated proteins have been found associated with macrophages and inflammation (by using an anti-nitrotyrosine antibody) in atheromatous plaques in human vessels (Beckman, J.S., *et al.*, *Biol. Chem. Hoppe-Seyler 375*:81-88 (1994)), providing further evidence that nitrotyrosine is formed *in vivo* in humans.

SUMMARY OF THE INVENTION

The invention relates to a method of treating or preventing autoimmune diseases, such as rheumatoid arthritis, insulin dependent diabetes mellitus, systemic lupus erythematosus and glomerulonephritis, comprising administering, preferably enterally, to a patient in need thereof an effective amount of a nitric oxide synthase inhibitor or a nitric oxide scavenger.

BRIEF DESCRIPTION OF THE DRAWING

The Figure is a bar graph showing the scores of pathological characteristics in MRL-*lpr/lpr* mice either treated with N^G-monomethyl-L-arginine (NMMA) or left untreated.

DETAILED DESCRIPTION OF THE INVENTION

The level of nitric oxide has now been linked to the manifestation of autoimmune diseases, particularly chronic diseases, such as rheumatoid arthritis, insulin dependent diabetes mellitus, systemic lupus erythematosus and glomerulonephritis. The inhibitors of nitric oxide synthase, known to synthesize nitric oxide in vivo, or nitric oxide scavengers can be useful in the prevention or treatment of autoimmune diseases (Gilkeson et al., Arth. Rheum. 36 (Suppl.):S219, 1993 (September)).

Inhibitors of nitric oxide synthase which can be used in this invention are those known in the art and include substrate analogs, such as aminoguanidine, N^G-amino-L-arginine, N^G-methyl-L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, and N^G-iminoethyl-L-ornithine, flavoprotein binders, such as diphenylene iodonium, iodonium diphenyl and di-2-thienyl iodonium, calmodulin binders, such as calcineurin, trifluoroperazine, N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide and N-(6-aminohexyl)-1-naphthalenesulfonamide, heme binders, such as carbon monoxide, depleters and analogs of tetrahydrobiopterin, such as 2,4-diamino-6-hydroxypyrimidine, and induction inhibitors, such as corticosteroids, TGF-β^c-1, -2, 3, interleukin-4, interleukin-10 and macrophage deactivation factor (Nathan, The FASEB Journal, Vol. 6, Sept. 1992, pp. 3051-3064). Preferred are the substrate analogs of nitric oxide synthase, N^G-amino-L-arginine, N^G-methyl-L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, and N^G-iminoethyl-L-ornithine. Particularly preferred are N^G-amino-L-arginine, N^G-methyl-L-arginine, N^G-nitro-L-arginine, and aminoguanidine. Most preferred is N^G-methyl-L-arginine.

Pharmaceutically acceptable salts may also be administered. Examples of suitable salts include acid salts, such as hydrogen chloride, hydrogen bromide, hydrogen iodide, sulfate and acetate salts, as well as basic salts, such as amine, ammonium, alkali metal and alkaline earth metal salts.

Scavengers of nitric oxide are compounds which will react with nitric oxide in vivo, such as hemoglobin (Wang *et al.*, *Life Sciences 49*:55-60 (1991)) and cobalamins (Rajanayagam, C.G., *et al.*, *Brit. J. Pharmacol. 108*:3-5 (1993); Zatarain, J., *et al.*, *Clin. Res. 41*:783A (1993)).

The compounds described above are known in the art and are commercially available.

The compounds of the claimed invention can be administered alone or in a suitable pharmaceutical composition. Modes of administration are those known in the art, such as enteral, parenteral or topical application. Enteral is preferred and oral administration is particularly preferred.

Suitable pharmaceutical carriers can be employed and include, but are not limited to water, salt solutions, alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose or starch, magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, flavorants, coloring, and/or aromatic substances and the like which do not deleteriously react with the active compounds.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants,

including suppositories. Ampoules are convenient unit dosages. Oral applications are preferably administered in the forms of capsules, tablets and/or liquid formulations. Unit form dosages are preferred. Topical applications can be administered in the form of a liquid, gel or a cream.

It will be appreciated that the actual amounts of the active compounds in a specific case will vary according to the specific compound being utilized, the particular composition formulated, the mode of administration and the age, weight and condition of the patient, for example. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

The invention is further specifically illustrated by the following exemplification.

EXEMPLIFICATION

Nitrite/nitrate excretion

Mice were housed in metabolic cages (3 per cage) and fed deionized-distilled sterile water and a defined arginine and nitrate-free diet. Urine was collected into isopropanol to inhibit bacterial growth. Urinary nitrite/nitrate concentration was determined spectrophotometrically as described before (Granger, D.L. *et al.*, *J. Immunol. 146*:1294 (1991)). Determinations were done in duplicate or triplicate. Urinary protein was measured by the Bradford assay. Total nitrate excretion was then calculated based on the concentration and the urine volume.

In animals consuming a nitrate-free diet, urinary excretion of nitrite/nitrate accurately reflects the endogenous production of NO. To determine the production of NO in mice of different strains, we analyzed urine

collected daily under basal conditions. MRL-*lpr/lpr* mice excrete more nitrite/nitrate than do C3H mice, when analyzed over a 10 day period at age 3 months. Likewise, when analyzed over time beginning at age 6 weeks, MRL-*lpr/lpr* mice excrete higher levels of nitrite/nitrate than do *B6* mice as the mice age. Higher nitrate/nitrite excretion begins at approximately age 10 to 12 weeks paralleling that of proteinuria. Oral administration of 50 mM NMMA in water to the MRL-*lpr/lpr* mice reduced the high level nitrite/nitrate excretion. This signifies that the nitrite/nitrate is a product of NOS, since NMMA is a specific inhibitor of the enzyme. Levels of nitrite/nitrate excretion in 5 month old mice of strains MRL-+/+ (0.8 μmol per mouse per day) and B6-*lpr/lpr* (1.2 μmol per mouse per day) (3 mice in each group) were normal These results indicate that neither the *lpr* gene *per se* nor the MRL genetic background is adequate for the expression of enhanced nitrite excretion, and that both the *lpr* gene and genetic factors in the MRL background are necessary.

Nitric oxide production *in vitro* and nitric oxide synthase content

Spleen, liver, kidneys, lymph nodes, and peritoneal cells were collected and quickly frozen in a dry ice-ethanol slurry in a buffer-protease inhibitor cocktail containing 100 μM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 1 μg/ml chymostatin, and 5 μg/ml pepstatin A. The tissue cells were then disrupted with a pestle in repeated freeze-thaw cycles. Cytosol was collected after centrifugation, and assayed for protein and NOS activity using a modification of procedures known previously (Bredt, D.S. and Snyder, S.H., *Proc. Natl. Acad. Sci. USA 86*:9030 (1989); Sherman, P.A., *et al., Biochemistry 32*:11600 (1993)). The assay buffer contained 50 mM HEPES

(pH 7.5), 200 μM NADPH, 1 mM dithiothreitol, 10 μM FAD, 100 μM tetrahydrobiopterin, and 10 μM L-arginine. We used L-arginine labeled with tritium in the guanidino position (product number NEC453, New England Nuclear, Wilmington, DE). Thirty microliters of sample were used in a total reaction mixture of 50 microliters. Samples were done in duplicate or triplicate.

Peritoneal macrophages from the normal BALB mice had no enhancement of nitrite/nitrate production when treated with endotoxin or IFN-γ alone, but the combination enhanced the production greatly. In contrast, peritoneal macrophages from MRL-*lpr/lpr* mice had enhanced responses to treatment with endotoxin and murine IFN-γ alone, as well as with combined endotoxin IFN-γ treatment. To determine if there was enhanced tissue iNOS mRNA expression, RNA was extracted from organs from BALB and MRL-*lpr/lpr* mice, and then examined by Northern analysis for iNOS mRNA expression. The iNOS mRNA (approximately 4.7 kilobases in size) was noted in tissue from kidney and spleen from MRL-*lpr/lpr* mice, but not in those tissues from BALB mice. Various tissues and cells from MRL-*lpr/lpr* and BALB mice were extracted and analyzed for their abilities to convert ^{14}C-L-arginine (labeled in the guanidino position) to ^{14}C-L-citrulline. Peritoneal macrophages and spleen extracts from MRL-*lpr/lpr* mice displayed more NOS activity than did those from BALB mice, while the activity in kidney extracts was not different.

By immunofluorescence analysis using a rabbit anti-mouse iNOS antibody, we noted no NOS antigen expression in spleen, liver, and kidney from BALB mice, and none in liver or kidney from MRL-*lpr/lpr* mice. However, spleens from MRL-*lpr/lpr* mice displayed large numbers of cells containing the NOS antigen. Comparable findings were noted when we used a monospecific guinea pig

anti-rat inducible NOS antibody. When tissue extracts were analyzed for iNOS antigen by immunoprecipitation and immunoblotting techniques using a rabbit anti-mouse iNOS antibody, we did not detect antigen in extracts from organs of BALB mice, although extracts from spleen and kidney tissues from MRL-*lpr/lpr* mice had readily detectable antigen.

NMMA treatment

Groups of 8 week old MRL-*lpr/lpr* mice were given either sterile, distilled deionized water or water containing 50 mM NMMA for *ad libitum* consumption. NMMA was from CalBiochem (San Diego, CA) and from Dr. Owen Griffith (Milwaukee, WI). Both groups of mice were maintained on the defined nitrate free diet described above. At weekly intervals, the mice were placed in metabolic cages and 24 hour urine collections were obtained. Urinary nitrite/nitrate was measured as described above, and urinary protein was determined using the Bradford assay (BioRad, Hercules, CA). After 10 weeks of treatment, the mice were bled and sacrificed with removal of the kidneys and knee joints.

Serum anti-DNA activity was determined by ELISA as previously described. The kidneys were imbedded in paraffin, sectioned and stained with hematoxylin and eosin. Knee joints were decalcified in folic acid, embedded into paraffin, sectioned, and stained. Slides were then read by a pathologist "blinded" as to the group of origin. The amount of kidney and knee joint disease present in each specimen was quantitated as noted before. Briefly, glomeruli were graded for hypercellularity (0-4), hyperlobularity (0-4), crescents (0-4), and necrosis (0-4). A score was then derived by adding the grading of these features of glomerular disease. Kidneys from normal BALB mice usually have scores from 0-1. Vasculitis was

noted when present in medium size vessels in the kidney sections. The synovial score was derived by adding the grading of synovial proliferation (0-3) and subsynovial inflammation (0-3). Knee joints from normal BALB mice usually have scores from 0-0.5.

Groups of MRL-*lpr/lpr* mice received either double distilled water (n=10) or water containing 50 mM NMMA (n=9) *ad libitum* beginning at 8 weeks of age. Both groups of mice received the defined nitrate free diet. Mice were treated for a total of 10 weeks. Mice in both groups appeared clinically normal. However, two mice in the NMMA group died during week 3 of treatment leaving 7 mice in the NMMA group for analysis. Extensive autopsies (including careful histological examinations and culturing of serum, urine, and organs) on these two mice and on a comparable mouse that had received NMMA 4 weeks revealed no evidence of microbial infection or other evident cause of death. Administration of NMMA in the drinking water of MRL-*lpr/lpr* mice effectively blocked nitrite/nitrate excretion (and by inference nitric oxide production). Also, mice receiving NMMA excreted significantly less protein than did control mice; this difference became apparent at week five of treatment.

Pathologic examination of the kidneys and knee joints of mice from the two groups of mice revealed significantly less disease in the NMMA-treated group. Renal disease as measured by the renal score, and arthritis as measured by the synovial score were both significantly less in the NMMA group as compared to the control group. The observed differences for joint disease and renal disease are statistically different ($p<0.05$ and $p<0.02$, respectively, using the Mann-Whitney U test), while that for anti-DNA antibody level is not. There was minimal to no glomerular proliferation in mice treated with NMMA, while all but one of the control mice had significant glomerular

proliferation and hyperlobulation. The chronic interstitial lymphocytic infiltrate seen in the kidneys of all *lpr* congenic mice (including C3H-*lpr/lpr* mice that do not develop glomerulonephritis) was present to comparable degrees in both control and NMMA treated mice. Medium vessel vasculitis appears sporadically in the kidneys of untreated MRL-*lpr/lpr* mice with an overall incidence of 30%. Mild to moderate (1-2+) medium vessel vasculitis was present in 3/10 kidneys from mice in the control group. Mild vasculitis was seen in 1/7 kidneys from the NMMA treated group. There was not a statistical difference in vasculitis between the two groups, but the small numbers of mice with vasculitis makes it difficult to draw firm conclusions regarding the effects of NMMA on this aspect of inflammation. Synovial proliferation was significantly decreased in the mice treated with NMMA. While only 3 of 7 mice in the NMMA-treated group had abnormal knee joints with mild to moderate synovial proliferation and synovial inflammation, all 10 mice in the control water group had some degree of synovial proliferation and inflammation. Levels of serum anti-double stranded DNA measured at age 18 weeks in the two groups were essentially equivalent (see the Figure).

Formation of nitroso-hemoglobin

NO-Hb forms through an interaction of NO with iron in the heme group of hemoglobin (Huot, A.E., *et al.*, *Biochem. Biophys. Res. Commun.* *182*:151-157, (1992); Cantilena, L.R.J., *et al.*, *J. Lab. Clin. Med.* *120*:902-907 (1992)). Whole blood from MRL-*lpr/lpr* mice at different ages was analyzed for the presence of nitroso-hemoglobin (NO-Hb). The blood samples were anticoagulated and examined by electron paramagnetic resonance (EPR) at 77°K using a Bruker ESP300 spectrometer (Chamulitrat, W. *et al.*, *Molec. Pharmacol.* *46*:391-397 (1994)). Table 1 shows the mean ±

SEM EPR units (n=number of animals examined). An age-dependent increase was observed in the amount of NO-Hb in the blood of the diseased mice. The levels of NO-Hb were higher in MRL-*lpr/lpr* mice compared to same-age control mice without disease. The differences were statistically significant ($p<0.05$ at all ages analyzed). The presence of NO-Hb is another important sign that NO is being over-expressed in these mice with autoimmune nephritis and arthritis.

Table 1

Mouse	Age 12 weeks	Age 16 weeks	Age 18 to 20 weeks
MRL-*lpr/lpr*	1420 ± 130 (n=16)	2045 ± 226 (n=10)	4092 ± 711 (n=711)
BALB/c (control)	970 ± 221 (n=5)	1005 ± 160 (n=160)	811 ± 108 (n=8)

The above studies demonstrated an NO-mediated modification of a protein (hemoglobin) in these mice. To determine if diseased tissue is modified by NO in MRL-*lpr/lpr* mice, kidneys from normal BALB/c mice (20 weeks old) and MRL-*lpr/lpr* mice (20 weeks old) were examined by electron paramagnetic resonance (EPR). For these tissues, the spectrum of the control mouse was subtracted from that of the MRL-*lpr/lpr* mouse, and the resultant curve showed an easily detectable NO-non-heme iron tyrosyl signal at g=2.04, as well as the typical spectra for NO-heme (presumably due to blood trapped within the MRL-*lpr/lpr* kidney; see Chamulitrat, W., *et al., Molec. Pharmacol. 46*:391-397 (1994)). It is important to note that control kidney did not have these NO-protein signals, signifying that the nitrosylated

non-heme protein might be causally related to the observed severe renal pathology in these mice.

Protein nitration in kidneys from MRL-*lpr/lpr* mice

As noted above, NO may react with superoxide and form the highly reactive, tissue destructive molecule peroxynitrite. It has been shown previously that cells from MRL-*lpr/lpr* mice can overproduce reactive oxygen species such as hydrogen peroxide, superoxide (Dang-Vu, A.P. *et al.*, *J. Immunol. 138*:1757-1761 (1987)) and nitric oxide (Weinberg, J.B., *et al.*, *J. Exp. Med. 179*:651-660 (1994)). A study was done to look for evidence that MRL-*lpr/lpr* mice also overproduce the destructive molecule peroxynitrite (Beckman, J.S., *et al.*, *Methods Enzymol. 233*:229-240 (1994)).

Since peroxynitrite causes nitration of tyrosine residues in proteins, a mono-specific, polyclonal anti-tyrosine antibody was used to detect evidence of the presence of peroxynitrite in diseased kidneys of MRL-*lpr/lpr* mice. Immunoblot analysis was performed on protein extracts from kidneys of 20 week old normal (BALB/c) and MRL-*lpr/lpr* mice.

Kidney tissue was homogenized with a glass pestle. Proteins in soluble extracts (100 μg per lane) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (0.45 μm, Novex). Unbound sites were blocked by incubation with 1% non-fat dry milk in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 60 min at 25°C. Membranes were incubated overnight at 25°C with a polyclonal anti-nitrotyrosine antibody (0.25 μg/ml) in 1% milk/TBS. Immunoreactivity was visualized by incubation with goat anti-rabbit IgG-HRP conjugate (Bio-Rad) (1:2000) dilution in 1% milk/TBS, followed by enhanced chemiluminescence (ECL) detection (Amersham).

While the immunoblot from kidneys from four individual control mice showed only one protein band reacting with the antibody, the immunoblot from kidneys from MRL-*lpr/lpr* mice showed this same band plus four other prominent bands. This indicates that *in vivo*-generated NO in MRL-*lpr/lpr* mice is converted to peroxynitrite, and that this peroxynitrite subsequently nitrates tyrosine residues in proteins in the kidneys.

Catalase activity in kidneys of MRL-*lpr/lpr* mice

The catalase content in kidneys from 20 week old normal (BALB/c) and MRL-*lpr/lpr* mice was analyzed. Peroxynitrite or NO can destroy catalase activity. Catalase was measured by the disappearance of hydrogen peroxide noted by absorbance at 240 nm (Beers, R.F., and Sizer, R.W., *J. Biol. Chem. 195*:133 (1952)). Values shown in Table 2 are the mean ± SEM of replicate samples expressed in units/mg protein. Table 2 shows that kidneys from the control mice had high levels of catalase while levels of catalase from MRL-*lpr/lpr* mice were very low.

Table 2

BALB/c mouse		MRL-*lpr/lpr* mouse	
Mouse 1	77 ± 6	Mouse 1	12 ±1
Mouse 2	75 ± 2	Mouse 2	20
Mouse 3	67 ± 5		
Mouse 4	68 ± 6		

In a different type of analysis to determine catalase, we studied protein extracts from kidneys by

PAGE, with subsequent visualization of catalase activity by soaking the gels in phosphate buffer containing horseradish peroxidase and hydrogen peroxidase with diaminobenzidine.

Soluble extracts (25 μg/lane) were separated by electrophoresis on a 6% native polyacrylamide gel. Bands of catalase activity were visualized by soaking the gels for 45 min at 25°C in 50 mM sodium phosphate, pH 7.0, containing 0.1 mM EDTA and 50 μg/ml horseradish peroxidase. H_2O_2 was added to a final concentration of 5.0 mM and the gel was incubated an additional 15 min. After a brief rinse in water, stain development was initiated by addition of 0.5 mg/ml diaminobenzidine-HCl in 50 mM sodium phosphate, 0.1 mM EDTA, pH 7.0.

Results showed clearly that kidneys from 4 different BALB/c control mice contained large amounts of catalase, while those from two MRL-*lpr/lpr* mice had markedly diminished levels of catalase. However, if the mice had been treated with oral N^G-monomethyl-L-arginine *in vivo* (See NMMA treatment above), the catalase level in kidneys from three different MRL-*lpr/lpr* mice was normal (comparable to that of the control mice). This signifies that catalase activity (which may be inhibited by the actions of NO or peroxynitrite) is markedly low in MRL-*lpr/lpr* mice, and that the NO- (or peroxynitrite-) mediated decrease in catalase is blocked by *in vivo* administration of N^G-monomethyl-L-arginine.

Human investigations

To determine if humans with arthritis have iNOS protein expressed in their synovial tissues, these tissues were studied by immunofluorescence techniques using mouse monoclonal anti-iNOS antibody (purchased from Transduction Laboratories, Inc.). Of six synovial samples removed from

patients with advanced arthritis at the time of joint replacement surgery, iNOS antigen was detected in the tissues of two of three rheumatoid arthritis patients and in one of three osteoarthritis patients. These studies demonstrate that patients have synovia that contain iNOS, and that it may be overexpressed in synovia of patients with rheumatoid arthritis.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described specifically herein. Such equivalents are intended to be encompassed in the scope of the following claims.

CLAIMS

1. A method of treating or preventing autoimmune diseases in a patient comprising administering to the patient an effective amount of a nitric oxide synthase inhibitor.

2. A method of claim 1 wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, insulin-dependent diabetes mellitus, systemic lupus erythematosus and glomerulonephritis.

3. A method of claim 2 wherein the autoimmune disease is rheumatoid arthritis.

4. A method of claim 3 wherein the mode of administration is enteral.

5. A method of claim 3 wherein the mode of administration is parenteral.

6. A method of claim 4 wherein the nitric oxide synthase inhibitor is selected from a group consisting of N^G-amino-L-arginine, N^G-methyl-L-arginine, N^G-nitro-L-arginine, N^G-nitro-L-arginine methyl ester, N^G-iminoethyl-L-ornithine and aminoguanidine.

7. A method of claim 6 wherein the nitric oxide synthase inhibitor is N^G-methyl-L-arginine.

8. A method of treating rheumatoid arthritis in a patient in need of treatment thereof comprising administering to said patient an effective amount of N^G-methyl-L-arginine.

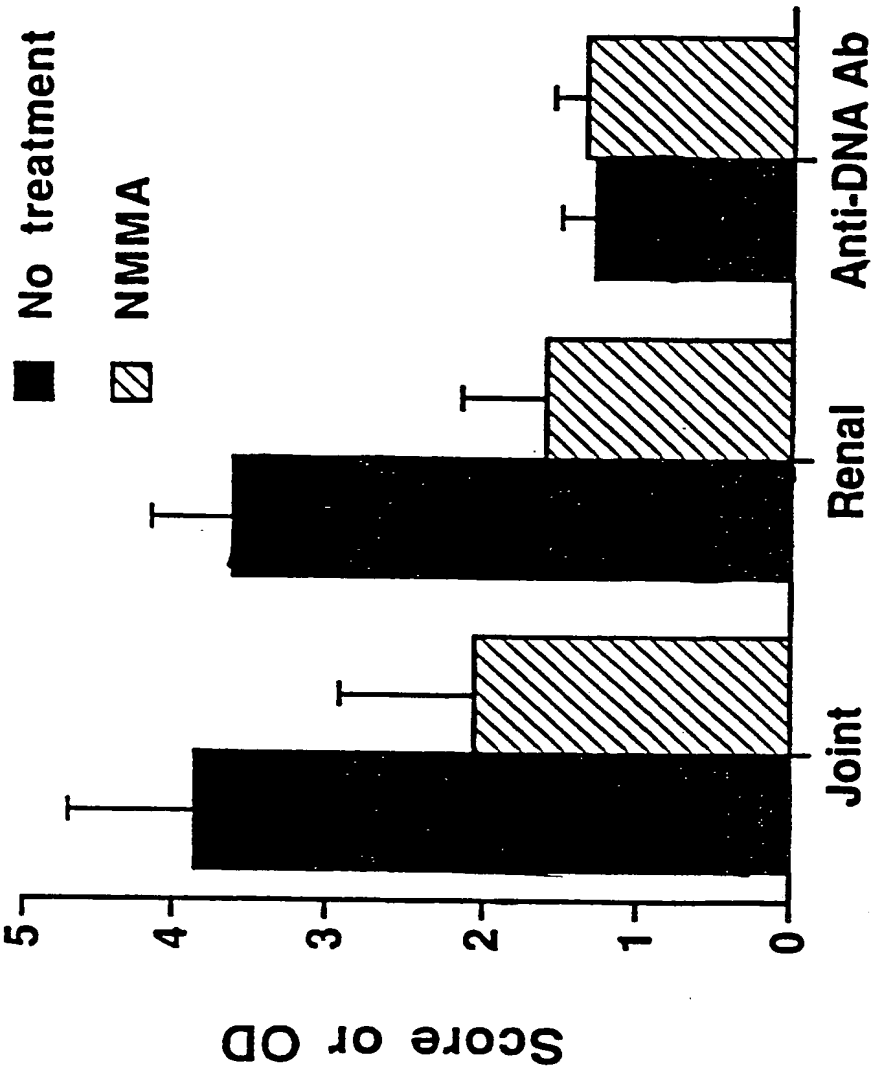
9. A method of treating or preventing autoimmune diseases in a patient comprising administering to the patient an effective amount of a nitric oxide scavenger.

10. A method of claim 9 wherein the autoimmune disease is selected from the group consisting of rheumatoid arthritis, insulin-dependent diabetes mellitus, systemic lupus erythematosus and glomerulonephritis.

11. A method of claim 10 wherein the autoimmune disease is rheumatoid arthritis.

12. A method of treating rheumatoid arthritis in a patient in need of treatment thereof comprising administering to said patient an effective amount of N^G-methyl-L-arginine.

13. A nitric oxide synthase inhibitor for use in therapy, for example for use in treating or preventing an autoimmune disease.


No treatment
NMMA
Anti-DNA Ab
Renal
Joint
0
1
2
3
4
5
Score or OD

INTERNATIONAL SEARCH REPORT

Internat l Application No
PCT/US 94/13239

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/22 A61K31/195 A61K31/155

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BR. J. PHARMACOL., vol.110, no.2, October 1993 pages 701 - 706 A. IALENTI 'Modulation of adjuvant arthritis by endogenous nitric oxide' see the whole document ---	1-6, 9-11,13
X	BIOCHEM BIOPHYS RES COMMUN (UNITED STATES), MAR 29 1991, VOL. 175, NO. 3, PAGE(S) 752-8, Kroncke KD et al 'Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation.' see the whole document --- -/--	1,2,4-7, 9,10,13

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 March 1995	10. 04. 95
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Authorized officer Stierman, B

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DIABETES (UNITED STATES), AUG 1992, VOL. 41, NO. 8, PAGE(S) 897-903, Corbett JA et al 'Does nitric oxide mediate autoimmune destruction of beta-cells? Possible therapeutic interventions in IDDM.' see the whole document	1,2,4-7, 9,10,13
X	BIOCHEM BIOPHYS RES COMMUN (UNITED STATES), JUN 30 1993, VOL. 193, NO. 3, PAGE(S) 1269-74, Nicolson AG et al 'Induction of nitric oxide synthase in human mesangial cells.' see the whole document	1,2,4-7, 9,10,13
X	BIOCHEM BIOPHYS RES COMMUN,, VOL. 178, NO. 3, PAGE(S) 913-920, 1991. LUKIC M L ET AL 'INHIBITION OF NITRIC OXIDE GENERATION AFFECTS THE INDUCTION OF DIABETES BY STREPTOZOCIN IN MICE' see the whole document	1,2,4-7, 9,10,13
X	EP,A,0 547 558 (WASHINGTON UNIVERSITY) 23 June 1993 see the whole document	1-6, 9-11,13
X	WO,A,91 04023 (CORNELL RESEARCH FOUNDATION) 4 April 1991 see the whole document	1,4-6,9, 13
X	WO,A,93 13055 (THE WELLCOME FOUNDATION LTD.) 8 July 1993 see the whole document	1-6, 9-11,13
X	AM J PATHOL (UNITED STATES), NOV 1991, VOL. 139, NO. 5, PAGE(S) 1047-52, COOK HT ET AL 'Glomerular nitrite synthesis in in situ immune complex glomerulonephritis in the rat.' see the whole document	1,2,4-7, 9,10,13
X	26TH ANNUAL MEETING OF THE ASN (AMERICAN SOCIETY OF NEPHROLOGY), BOSTON, MASSACHUSETTS, USA, NOVEMBER 14-17, 1993.;& J AM SOC NEPHROL,, VOL. 4, NO. 3, PAGE(S) 610, 1993. KETTELER M ET AL 'AMINOGUANIDINE INHIBITS NO-SYNTHESIS IN NEPHRITIC GLOMERULI' see abstract	1,2,4-7, 9,10,13

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ARTHRITIS AND RHEUMATISM, vol.36, no.9, September 1993 page S44 M. STEFANOVIC-RACIC 'N-Monomethylarginine, an inhibitor of nitric oxide synthase, suppresses the onset of adjuvant arthritis in rats'	1-13
Y	see abstract ---	1-12
X	BRIT. J. PHARMACOL., vol.107, 1992 pages 1159 - 1162 M.S. MULLIGAN 'Protective effects of inhibitors of nitric oxide synthase in immune complex-induced vasculitis' cited in the application	1,4-7,9, 13
Y	see the whole document ---	2,10
X	J. EXP. MED., VOL. 178, PAGE(S) 749-54, August 1993 MCCARTNEY-FRANCIS, NANCY ET AL 'Suppression of arthritis by an inhibitor of nitric oxide synthase' see the whole document ---	1-13
Y	ANN. RHEUM. DIS., vol.51, 1992 pages 1219 - 1222 A.J. FARRELL 'Increase concentrations of nitrite in synovial fluid and serum samples suggest increased nitric oxide synthesis in rheumatic diseases' cited in the application see the whole document ---	1-12
Y	IMMUNOBIOLOGY, vol.189, no.1-2, September 1993 pages 116 - 117 IOSSIFIDOU K ET AL 'Assessment of mesangial cells isolated from normal and from SLE prone mice' see the whole document ---	2,10
Y	ARTH. RHEUM., vol.36, September 1993 page S219 G. GILKESON ET. AL. 'Nitric oxide is spontaneously produced in MRL-lpr mice with overproduction paralleling clinical disease' cited in the application see abstract --- -/--	1-12

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BRIT. J. PHARMACOL., vol.108, no.1, January 1993 pages 3 - 5 M.A.S. RAJANAYAGAM 'Differential effects of hydroxocobalamin on NO-mediated relaxations in rat aorta and anococcygeus muscle' cited in the application see abstract	9-11
X,Y	AU,B,5 453 490 (M. ARLINGTON) 8 November 1990 see claims 19,22,26,31	9-11

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/US94/13239

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-12 are directed to a method of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 1-5, 9-11, 13
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
Please see attached sheet.

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Obscurities, Inconsistencies....

Expressions like "autoimmune diseases", "nitric oxide synthase inhibitor", "nitric oxide scavenger" do not make sufficiently clear which diseases or specific compounds are meant. The search had therefore to be restricted to the diseases and compounds expicitly mentioned in the claims and to the general inventive concept.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interna l Application No
PCT/US 94/13239

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0547558	23-06-93	CA-A-	2085399	17-06-93
		JP-A-	5255079	05-10-93
		US-A-	5358969	25-10-94
		US-A-	5246971	21-09-93
		US-A-	5246970	21-09-93
WO-A-9104023	04-04-91	US-A-	5059712	22-10-91
		EP-A-	0491708	01-07-92
		JP-T-	5500659	12-02-93
		US-A-	5158883	27-10-92
WO-A-9313055	08-07-93	AU-B-	3169293	28-07-93
		EP-A-	0618898	12-10-94
AU-B-5453490		NONE		